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ORIGINAL PAPER

Effect of elicitors on the production of gossypol and methylated gossypol in cotton hairy roots

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Abstract The effect of two chemical elicitors, salicylic acid and methyl jasmonate, on the production of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol in *Gossypium barbadense* hairy roots was examined. Methyl jasmonate, but not salicylic acid, was found to increase the production of gossypol and its methylated forms, but with a concomitant reduction in culture growth. The optimal methyl jasmonate dose was between 100 and 300 μM for hairy roots harvested 7 days after elicitation. After 20 d of induction with 100 μM methyl jasmonate, an eightfold increase in the level of gossypol was observed in elicited cultures compared with control cultures, double the highest gossypol levels previously reported for any cotton tissue. A two to threefold increase in the level of 6-methoxygossypol and a slight increase in the levels of 6,6′-dimethoxygossypol were also observed. Although methyl jasmonate stimulated the production of both optical forms of gossypol, the distribution of the enantiomers was different between elicited and control cultures.

Keywords Cotton · Hairy roots · *Gossypium* · Gossypol · Methyl jasmonate · Salicylic acid

Abbreviations
MeJ Methyl jasmonate
SA Salicylic acid

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Introduction

Although rich in edible oil and high-quality protein, cottonseed also contains the phytoalexin gossypol (Fig. 1), a terpene aldehyde that can be toxic to human and monogastric animals. The presence of gossypol and related compounds currently hampers the use of cottonseed as a source of food (Mao et al. 2006); however, these same compounds have promising uses in medicine and agriculture. Gossypol possesses anticancer, antimicrobial, antiviral, antiparasitic, insecticidal and nematicidal activities and also has been used as a male contraceptive (Vander Jagt et al. 2000; Dodou 2005). Gossypol also greatly increases the efficacy of a variety of anticancer agents by interacting with pro-apoptotic proteins, detoxification enzymes and signaling kinases (Dodou 2005; Xu et al. 2005). Gossypol exists as a dimer of two-bridged naphthalene moieties that form enantiomers because of restricted rotation about the bridge bond (Fig. 1). The (−)-optical form of gossypol usually exhibits higher biological activity than the (+)-optical form (Dodou 2005). In an effort to find high-value uses for gossypol, our laboratory is engaged in extending and promoting gossypol research including efforts to improve the production of the compound and the preparation of natural and synthetic gossypol derivatives.

Although gossypol is routinely isolated from cottonseed, a particularly simple extraction procedure is possible from cotton roots (Royce et al. 1941). In addition to gossypol, roots also produce two related compounds, 6-methoxygossypol and 6,6′-dimethoxygossypol that could similarly be used to produce novel derivatives (Stipanovic et al. 1975; Dowd and Pelitire 2006). Production of these compounds in hairy root cultures would be advantageous as it would simplify their extraction, allow for production under controlled conditions, and potentially eliminate the

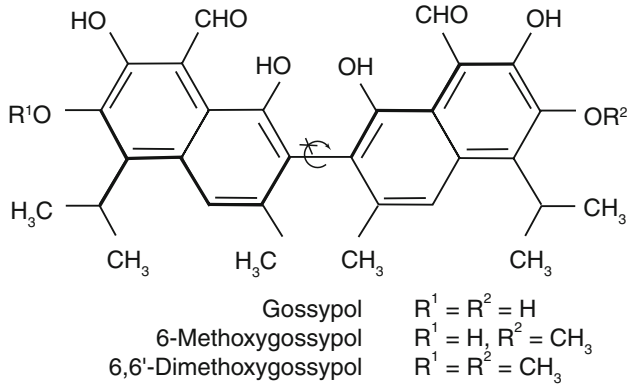

Fig. 1 Structure of gossypol depicted in the *S*-(+)-enantiomeric form. Substitution at the 6- and 6′-phenolic positions yields the mono- and di-methylated gossypol forms

variable levels of these compounds that are often found with native root or seed tissue.

Hairy roots are formed when plant tissues are inoculated with the bacterium *Rhizobium rhizogenes*. The plant tissues are transformed by the *Ri* (root inducing) plasmid from the bacteria, causing genetically stable adventitious roots to grow from the infection sites, which can be excised and grown independently on hormone-free solid and liquid medium containing sugar, a suitable nitrogen source, and appropriate minerals and vitamins (Guillon et al. 2006). Hairy roots can be continuously sub-cultured by transferring pieces from the growing mass to fresh medium. Like native roots, hairy roots produced from cotton varieties have proved to reliably produce gossypol and its two methylated derivatives (Triplett et al. 2008).

Elicitor molecules such as salicylic acid (SA) and methyl jasmonate (MeJ) are known to induce the production of secondary metabolites when added to culture medium (Shanks and Morgan 1999) and promote the production of terpenes (Peñuelas et al. 2007) including taxol (Yuan et al. 2002; Wang et al. 2004). Because gossypol is present in cotton hairy roots at levels roughly equivalent to those found in field-grown cotton roots (Triplett et al. 2008), we hypothesized that elicitor-induced secondary metabolite production in cotton hairy roots would be a promising source of even greater levels of these compounds.

In this paper, the effect of two commonly used phytochemical elicitors, SA and MeJ, on the production of gossypol, 6-methoxygossypol and 6,6′-dimethoxygossypol in a cotton hairy root culture line was studied. In addition, we determined the optimum dosage of the elicitors and studied the effect of harvest time of elicited roots to maximize gossypol yield. We also examined the effect of elicitors on the distribution of the individual gossypol enantiomers.

Materials and methods

Culture of hairy roots

The experiments were conducted with a hairy root clone originally obtained from the cotyledon leaves of *Gossypium barbadense* St. Vincent Sea Island Superfine cotton (GRIN PI 528406) infected with *Rhizobium rhizogenes* (ATCC 15834). The clone was from our initial study and was known to produce gossypol and its methylated derivatives (Triplett et al. 2008). Explants for the elicitor studies were taken from root masses that had been grown and subcultured continuously in liquid culture for several months before the start of the study. To begin each experiment, four root tips between 1.5 and 2.5 cm in length were inoculated into 6-well culture plates (USA Scientific, Ocala, FL) containing 5 ml of filter-sterilized Gamborg's B5 Basal Medium with Minimal Organics (Sigma–Aldrich, St. Louis, MO; G5893) that was supplemented with 20 g l^{-1} sucrose (Gamborg B5 + sucrose). The well lids were secured with Micropore surgical tape (3 M Co., St. Paul, MN) and root pieces were allowed to grow in darkness at 28°C at 100 rpm in a model G-24 orbital shaker (New Brunswick Scientific, Edison, NJ). The starter cultures were transferred to fresh plates containing new medium every 3 weeks. Once the roots had grown into a sizeable mass (~1 g fresh weight, usually in 3–6 weeks) they were transferred to autoclaved jars (6 cm diameter × 9 cm height) with Magenta B caps (Magenta Corp., Chicago, IL) containing 20–25 ml of filter-sterilized Gamborg B5 + sucrose. The jars were incubated in a darkened Innova 44 Incubator Shaker (New Brunswick Scientific Co., Edison, NJ) at 28°C and 100 rpm for the duration of the study.

MeJ and SA dosage study

SA was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ) and MeJ was purchased from Sigma–Aldrich Co. (St. Louis, MO). Filter-sterilized stock solutions of each elicitor were made in 95% ethanol at concentrations from 10 mM to 1.0 M. Ten days after transfer of the hairy roots to jars containing 25 ml fresh Gamborg B5 + sucrose, elicitor stock solutions (25 µl) were added to five replicate cultures to yield final concentrations of 0, 10, 100, 300, 500 and 1,000 µM for MeJ and 0, 10, 100, 500 and 1,000 µM for SA. Control replicates received 25 µl of 95% ethanol. The roots were allowed to grow in the presence of the elicitor for 7 days before harvesting. Root masses were weighed and stored at −70°C before freeze-drying and subsequent gossypol analysis.

Time-course study

MeJ, but not SA, stimulated gossypol and methylated gossypol production in cotton hairy roots. Forty-four replicate hairy root cultures were initiated to determine the time course of MeJ induction. After reaching approximately 1 g fresh weight, the starter cultures were transferred to fresh medium and allowed to grow for 2 d before addition of elicitor. MeJ (25 µl) was added to 20 jars to yield a final concentration of 100 µM (treatment). For a control, 20 jars received 25 µl of 95% ethanol. In addition, four root samples were harvested to determine the values of fresh and dry weight and gossypol content of the roots at time zero. Four control and four treatment hairy root cultures were harvested thereafter on days 2, 5, 10, 15 and 20. Harvesting the hairy roots consisted of removing the culture from the containers with forceps, gently blotting the hairy root mass between a folded paper towel to remove excess medium, measuring the fresh weight, and placing the sample in a 20-ml glass vial for storage at −70°C. In addition, 4 ml of the medium was also sampled and stored in 20-ml glass vials, and the sections of paper towel used to blot the top and bottom of each root sample were also cut out and stored individually in glass vials at −70°C.

Gossypol analysis of hairy roots

Prior to analysis, frozen hairy root samples were freeze-dried and ground in a Wiley mill fitted with a size 10 mesh screen. Total gossypol was determined by HPLC, similar to the procedure described by Hron et al. (1990), except that 30-mg samples were used for the analysis. Briefly, ground hairy root material was measured into a test tube, suspended in 2 ml of a 2% 3-amino-1-propanol derivatizing reagent (3-amino-1-propanol:glacial acetic acid: *N*,*N*-dimethylformamide 2:10:88 v/v/v), and heated at 95°C for 30 min. 3-Amino-1-propanol reacts with the gossypol aldehyde groups to form a Schiff's base complex that can be measured by reverse-phase HPLC. After heating, the samples were allowed to cool, 8 ml of mobile phase (see below) was added, and the contents were thoroughly mixed. A 1.8-ml volume of each sample was transferred into a microcentrifuge tube and centrifuged at ~7,000*g* for 5 min. The clarified supernatant liquid was decanted into HPLC vials for analysis.

Chromatography was conducted with a Waters (Milford, MA) model 2685 pumping system, a Waters model 916 photodiode array detector, and an SGE (Austin, TX) Inertsil ODS-2 reverse phase column (5 µm particles, 4 mm i.d. × 100 mm length). The mobile phase consisted of 65% acetonitrile and 35% 10 mM KH_2PO_4 pH 3.0 buffer, and the mobile phase flow rate was 1.0 ml/min. Generally, injection volumes were 20 µl and run times were 10 min. Ultraviolet-visible absorption spectra were recorded from 210 to 700 nm for each peak and 254 nm was used to quantify the compounds. A standard curve was developed from racemic gossypol-acetic acid (89.64% gossypol) as described by Hron et al. (1990). Previously determined relative response factors were used to quantify the amounts of 6-methoxygossypol and 6,6′-dimethoxygossypol (Dowd and Pelitire 2006).

For the 10- and 20-d samples from the time course study, the amounts of the individual gossypol enantiomers were determined. This was achieved by substituting chiral *R*-(−)-2-amino-1-propanol for 3-amino-1-propanol as the complexing amine (Kim et al. 1996; Hron et al. 1999) and adjusting the mobile phase to 50% acetonitrile and 50% buffer to extend the HPLC run time and allow better separation of the resulting diastereomers. For these calculations, *R*-(−)-2-amino-1-propanol was also used with racemic gossypol-acetic acid to generate a standard curve for each Schiff's base diastereomer, and previously determined relative response factors were used to quantify the amounts the individual enantiomers of 6-methoxygossypol and 6,6′-dimethoxygossypol (Dowd and Pelitire 2008).

Analysis of medium and paper towel blots

Before gossypol analysis, both the paper towel blots and the liquid medium were freeze dried. The paper towel sections were cut into pieces and wedged into the base of a 12.3 cm × 2 cm diameter test tube and analyzed for gossypol compounds as above for root tissue, except that 4 ml of the 3-amino-1-propanol complexing reagent was added to each tube to cover all of the towel pieces, and samples were subsequently diluted with only 6 ml of mobile phase after heating. To analyze the gossypol content of the medium, the freeze-dried medium powder was first dissolved in 2.5 ml of the 3-amino-1-propanol complexing reagent. After dissolution, 2 ml was transferred into a test tube, and derivatization and analysis was carried out as described for the hairy root tissue.

Calculations and statistics

The amounts of gossypol, 6-methoxygossypol and 6,6′-dimethoxygossypol were expressed as percentages of the dry tissue. The distribution of gossypol enantiomers was expressed as the percentage of (+)-gossypol, i.e., the amount of (+)-gossypol divided by the amounts of (+)- and (−)-gossypol. Statistical analyses were carried out using analysis of variance (ANOVA) with the StatView software package (SAS Institute and Inc. 1999) with either dosage or treatment as the main effect. The Fisher's Protected Least Significant Difference (PLSD) test was used to

detect statistical differences between dosages in the SA/MeJ dosage study.

Results

Dosage study with SA and MeJ

After 7 days, SA significantly reduced the dry weights of cotton hairy roots in a dose dependent fashion ($P = 0.0418$) (Fig. 2a). The reduction in mass was greater than threefold between the untreated and 1.0-mM SA treatments. Fresh tissue weight showed a similar effect, although not statistically significant (Fig. 2a).

MeJ elicitation also reduced the fresh and dry weight of the roots after 7 days ($P = 0.0059$ and $P < 0.0001$, respectively); however, the magnitude of the effect was less severe with a 2- to 2.5-fold mass change between the control and 1.0-mM MeJ treatments (Fig. 2b). In addition, the effect did not appear as dose dependent at the lower concentrations; statistically significant reductions in mass only occurred at the 100, 500 and 1,000 μM concentrations (Fig. 2b).

SA did not affect the levels of gossypol, 6-methoxygossypol and 6,6′-dimethoxygossypol at any of the tested doses (data not shown); however, MeJ did elicit the production of gossypol and 6-methoxygossypol (Fig. 3). The weight percentage of each compound relative to hairy root dry weight increased significantly as the MeJ concentration increased from 0 to 300 μM, and then decreased as the MeJ concentration increased beyond 300 μM to 1.0 mM. The amount of gossypol produced in the presence of 100 and 300 μM MeJ was eightfold greater than the amount produced in the control samples, and the amounts of 6-methoxygossypol and 6,6′-dimethoxygossypol were around 2- and 1.3-fold greater, respectively (Fig. 3).

Time course study with MeJ

When hairy roots were treated with 100 μM MeJ and monitored over time, production of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol rose progressively in both the elicited and control cultures. Induced cultures, however, had higher gossypol and 6-methoxygossypol levels than did control values throughout the 20-d sampling period (Fig. 4). The level of 6,6′-dimethoxygossypol appeared to peak earlier, around day 15, and its level was not always significantly higher in the treated cultures than in the control cultures (Fig. 4). The levels of gossypol produced in MeJ-treated cultures were

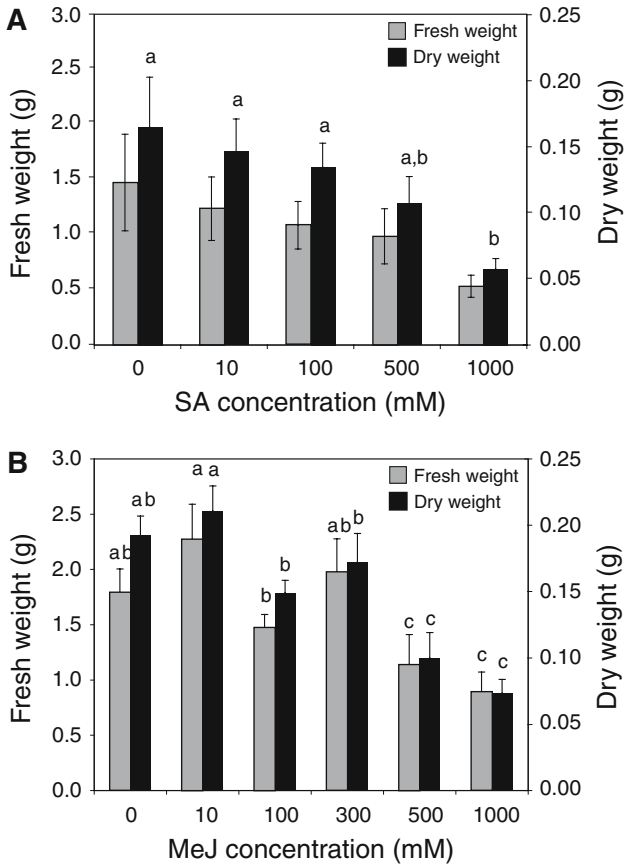

Fig. 2 Effect of chemical elicitors on the weight of cotton hairy root cultures harvested 7 days after the addition of elicitor. **a** SA significantly reduced the dry weights of the roots ($P = 0.04$). **b** MeJ significantly reduced the fresh ($P = 0.0059$) and dry ($P < 0.001$) weights of the roots. Different *lower case letters* denote significant differences in weights among dosage levels. *Error bars* represent standard errors

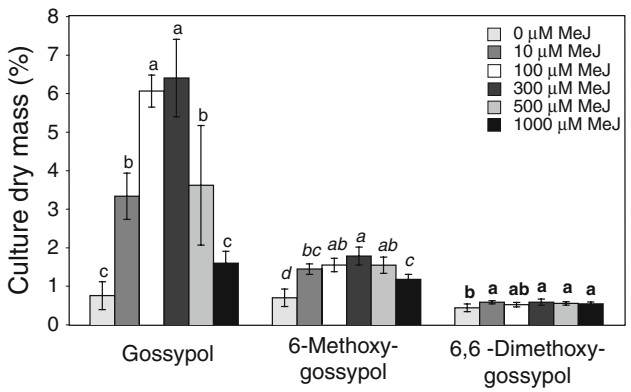

Fig. 3 Levels of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol 7 days after the addition of various doses of MeJ. A comparison of means was conducted for each compound. *Different letters* denote significant differences between dosages. *Regular letters* are used for gossypol; *italicized letters* are used for 6-methoxygossypol; and *bold letters* are used for 6,6′-dimethoxygossypol. *Error bars* represent standard errors

surprisingly high, around 10% at day 20, with some individual cultures having gossypol as greater than 12% of their dry mass.

As was observed in the concentration study, the addition of 100 μM MeJ also had a negative effect on tissue mass relative to the control cultures. This effect was apparent from around day 10 and increased progressively through day 20. At day 20, the MeJ-treated cultures weighed on average 36% less than did the control cultures.

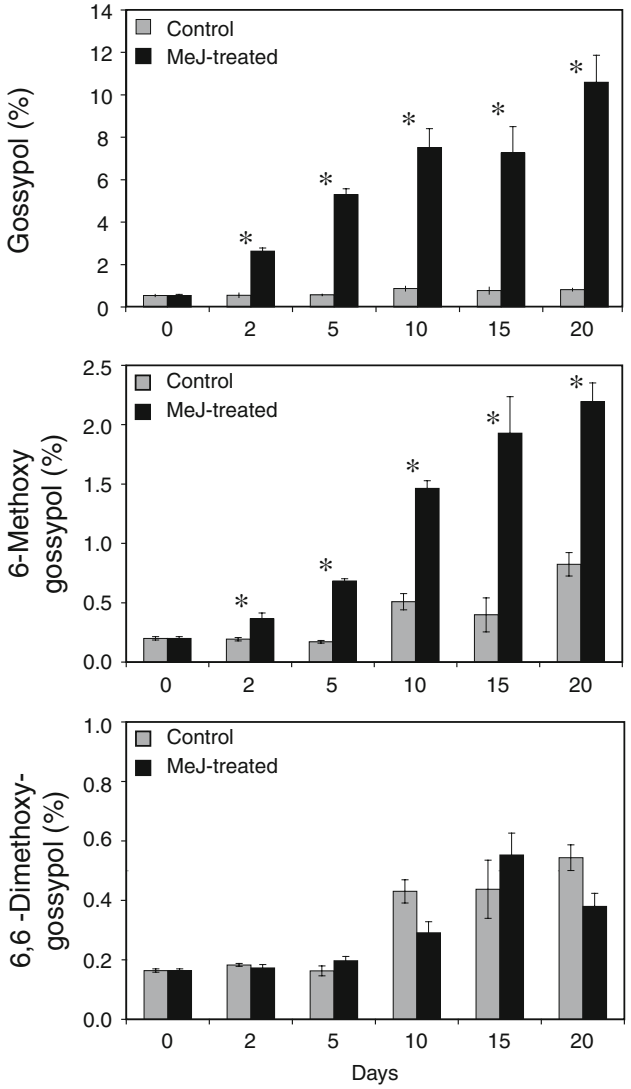

Fig. 4 Gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol levels over 20 days in control and MeJ-elicited cotton hairy root cultures. MeJ-treated samples produced significantly more gossypol ($P < 0.0001$) and 6-methoxygossypol ($P < 0.0001$) than did control samples. *Asterisks* represent statistically significant differences between treatments. *Error bars* represent standard errors

Analysis of gossypol compounds in culture medium and paper towel blots at 10 and 20 days

Cultures treated with MeJ appeared markedly more opaque than control cultures and contained many small particulates, suggesting that MeJ caused the shedding of cells or cellular debris from root surfaces. In addition, gentle blotting of hairy roots with tissue paper left behind a dark orange stain that was more pronounced in the MeJ-treated cultures, indicating that this material possibly contained gossypol and gossypol derivatives. Greater levels of gossypol were measured from the blots of cultures treated with MeJ compared with the blots of the control cultures (Fig. 5a). In addition, more of the gossypol compounds were measured in the medium of the MeJ-treated cultures compared with the medium of the controls (Fig. 5b). The increases in the levels of gossypol, 6-methoxygossypol and 6,6′-dimethoxygossypol detected corresponded roughly to the increased levels observed in the hairy root tissues. Although MeJ treatment resulted in more gossypol being detected in the medium and blotting paper, almost all of the gossypol and its two methylated forms was found within

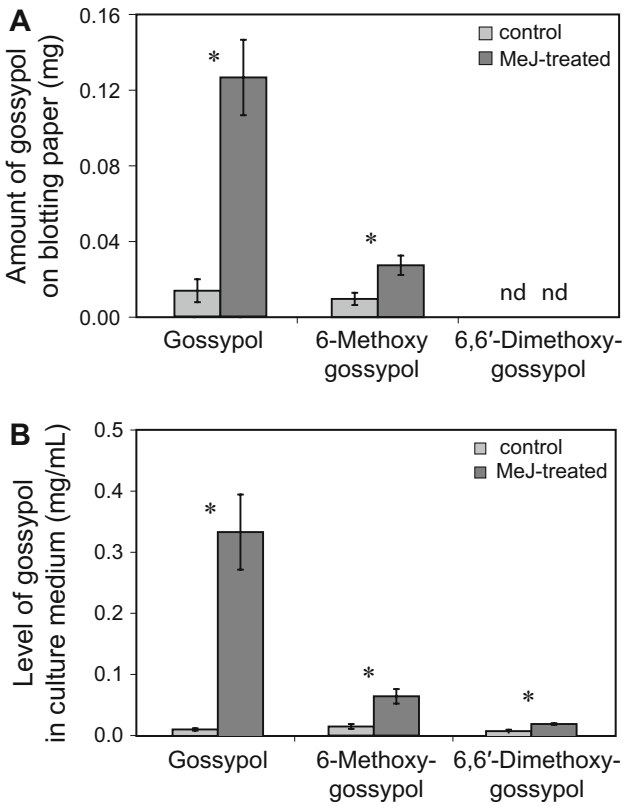

Fig. 5 Levels of gossypol, 6-methoxygossypol and 6,6′-dimethoxygossypol detected on blotting paper (used to remove excess medium) (**a**) and in the medium (**b**) of MeJ-elicited and control hairy root cultures. *Asterisks* represent statistically significant differences between the control and treated cultures. *Error bars* represent standard errors

Table 1 Distribution of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol (%) in the growth medium, tissue blots, and hairy roots at 10 and 20 days

Treatment—time/culture component analyzed	Gossypol	6-Methoxy-gossypol	6,6′-Dimethoxy-gossypol
Control cultures—10 day			
Hairy root mass	99.6	99.4	99.0
Blotting paper	0.1	0.2	0.4
Medium[a]	0.3	0.4	0.6
MeJ-treated cultures—10 day			
Hairy root mass	98.2	98.2	98.5
Blotting paper	0.2	0.2	0.4
Medium[a]	1.6	1.6	1.0
Control cultures—20 day			
Hairy root mass	99.9	99.8	99.9
Blotting paper	<0.1	<0.1	nd
Medium[a]	0.1	0.2	0.1
MeJ-treated cultures—20 day			
Hairy root mass	99.6	99.6	99.4
Blotting paper	<0.1	<0.1	nd
Medium[a]	0.4	0.4	0.6

[a] Gamborg's B5 Basal Medium with Minimal Organics and 20 g l^{-1} sucrose

Table 2 Percentage of the (+)-optical form of gossypol, 6-methoxygossypol, and 6,6′-dimethoxygossypol in cotton (*G. barbadense*, St. Vincent Sea Island Superfine) hairy root cultures reported as average ± standard deviation

Compound	Hairy roots, 10 d		Hairy roots, 20 d		Native seed[a]	Native root bark[a]
	Control	MeJ-treated	Control	MeJ-treated		
Gossypol	37.5 ± 5.5	56.8 ± 2.9	39.5 ± 3.0	56.1 ± 1.9	47.3	77.1
6-Methoxygossypol	42.1 ± 4.8	57.5 ± 2.9	47.2 ± 4.5	57.9 ± 2.3	46.5	67.1
6,6′-Dimethoxygossypol	48.2 ± 4.1	32.7 ± 6.6	49.3 ± 1.7	31.7 ± 5.0	45.5	58.6

[a] From Dowd and Pelitire (2008)

the recovered hairy root mass when the total mass of the compounds in the culture was calculated (Table 1).

Distribution of gossypol enantiomers in MeJ-elicited hairy root cultures

Considerable differences for the percentages of the individual enantiomers of the three compounds were observed in the hairy roots (Table 2). Both elicitation and methylation affected enantiomer distributions. However, no significant differences were observed between the 10- and 20-d data.

For the control cultures, the values of (+)-gossypol ranged between 38–40%, i.e., there was an excess of the (−)-gossypol form. The methylated gossypol forms tended to have higher levels of the (+)-optical form than did gossypol. For 6-methoxygossypol, 42–47% of the compound was in the (+)-form, whereas for 6,6′-dimethoxygossypol, approximately equal amounts of the two enantiomers were detected (Table 2).

MeJ-treated cultures differed in the ratios of enantiomers compared with the controls. For gossypol, the percentage of (+)-gossypol was 56–57%. Hence, there is an excess of the (+)-gossypol form in the MeJ-treated hairy roots, whereas there was an excess of (−)-gossypol in the control hairy roots. For 6-methoxygossypol, a similar pattern was observed. For 6,6′-dimethoxygossypol, the opposite trend was observed, with the MeJ-treated roots having between 32 and 33% of the (+)-optical form.

Discussion

SA and MeJ are two plant signaling molecules used to elicit the production of secondary metabolites such as anthraquinones (Bulgakov et al. 2002; Sirvent and Gibson 2002), flavonoids (Conceição et al. 2006), alkaloids (Avancini et al. 2003; Kang et al. 2004) glucosinolates (Li et al. 1999; Mikkelsen et al. 2003) and terpenes (Peñuelas et al. 2007) including the diterpene taxol (Yuan et al. 2002; Wang et al.

2004). In cotton hairy root cultures, SA did not increase the production of gossypol or its two methylated forms, at least at the time points and the concentrations tested. Xu et al. (2004) reported similar results, although they used a spectrophotometric method that quantifies the total pool of terpene aldehyes. Conversely, MeJ increased the levels of gossypol and its methylated derivatives. Other studies have demonstrated that MeJ upregulates transcription of the delta-cadinene synthase A gene that encodes the first enzyme in the gossypol biosynthetic pathway (Mao et al. 2006).

That MeJ and SA have different effects on the production of secondary compounds is not surprising, as the compounds operate in distinct plant signaling pathways and respond differently to different abiotic and biotic stresses (Pieterse and van Loon 1999). MeJ frequently induces the production of secondary metabolites, whereas SA varies in its effect on plant secondary metabolite biosynthesis. Both MeJ and SA increased the production of pilocarpine, an imidazole alkaloid, in 5-month-old *Pilocarpus microphyllus* seedlings (Avancini et al. 2003). Similarly, both MeJ and SA stimulated the production of the tropane alkaloid scopolamine in *Scopolia parviflora* hairy roots, and Western blot analysis showed that both SA and MeJ had a positive effect on the expression of the key biosynthetic enzymes, putrescine *N*-methyltransferase, and hyoscyamine (6*S*)-dioxygenase (Kang et al. 2004). In contrast, MeJ but not SA stimulated the production of the tropane alkaloid hyoscyamine in *Brugmansia suaveolens* hairy roots, and SA dampened the MeJ-stimulated production of the alkaloid (Zayed and Wink 2004). Therefore, the effect of SA on different plant species and varieties can vary even when comparing a single class of compounds such as alkaloids (Sirvent and Gibson 2002).

Both elicitors had a detrimental effect on the fresh and dry weight of cotton hairy roots. The effect of SA was proportional to its concentration, whereas the effect of MeJ appeared more complex, but it consistently reduced growth at concentrations exceeding 300 μM. SA is known to be phytotoxic (Durrant and Dong 2004), and the retardant effect on hairy root growth has been observed in other studies (Suresh et al. 2004; Kang et al. 2004). SA decreased the growth of *Atropa belladonna* hairy roots (Lee et al. 2001) and also caused cell death in *Taxus chinensis* var. *mairei* cultures with a concomitant release of ions and possibly other metabolites into the culture medium (Wang et al. 2007).

As gossypol can be inhibitory, it is likely that gossypol also contributes to reduced growth of the culture. Nevertheless, several points suggest that growth inhibition caused by gossypol is a secondary or minor effect. For example, although individual hairy root culture lines vary considerably in both their rate of growth and gossypol content (Triplett et al. 2008), analysis of a large population of cotton hairy root lines (from the Triplett et al. (2008) data) indicated that there was no correlation between these factors. In the current experiments, a similar lack of correlation was found in the MeJ dose experiment, where the greatest reduction in growth was found at the 1,000 μM concentration (Fig. 2b) but the greatest gossypol levels were present in the cultures grown in intermediate 100 and 300 μM concentrations (Fig. 3). In addition, the percent growth inhibition observed with cotton hairy roots tended to be roughly of the same order of magnitude as the inhibition reported in other non-gossypol-producing plant cultures (Kang et al. 2004; Suresh et al. 2004). Hence, although it is not possible to separate the effects of elicitor and gossypol, it appears that gossypol's contribution to the reduced growth rates of cotton hairy root cultures is secondary compared with the effect of the elicitors.

In most cotton plant tissues, including stems, leaves, and seeds, gossypol is found in lysigeneous glands that appear to serve as storages sites for these defense compounds. However, roots lack glands, and gossypol appears to be localized in the root bark of mature cotton and the epidermal cell layer of seedling roots (Mace et al. 1974; Stipanovic et al. 1975). This externalization is likely responsible for the relatively minor effect that gossypol appears to have on cotton hairy root growth rate. The mechanism of how gossypol reaches these external surfaces, either by external synthesis or internal synthesis and transport, are not well understood. Hunter et al. (1978) observed that either disturbing the roots of cotton seedlings or inoculating them with the pathogen *Rhizoctonia solani* also yielded greater levels of gossypol exudate. In this study, gossypol was detected in culture medium, tissue paper used to blot away excess medium off hairy roots, and along hairy roots, thus suggesting that gossypol was released and deposited along epidermal surfaces of these cultures. As higher levels of gossypol were detected in the medium and on blotting paper of MeJ-treated than control cultures, the mechanism responsible for deposition of gossypol along external culture surfaces was also enhanced in MeJ-treated hairy roots.

Since MeJ has been reported to elicit other terpenes, the increase the production of gossypol and its related compounds with MeJ treatment was not unexpected; however, the degree of elicitation was much greater than that reported in similar studies on other secondary metabolites. The 10–12% gossypol levels observed in the cotton hairy root cultures were double the highest levels previously reported for any *Gossypium* sp. tissue, e.g., the 6% value reported from incubated cotton root tips by Smith (1961) or the 5.7% value reported for *G. davidsonii* seeds by Stipanovic et al. (2005).

The MeJ-treated cultures appeared markedly more opaque and contained numerous small particulates, suggesting that MeJ caused cell death or, at least, contributed

to the shedding of cellular debris from root surfaces. Because gossypol is not readily soluble in aqueous medium, it is likely that the additional gossypol found in the medium was associated with this suspended material.

6-Methoxygossypol levels also increased by the MeJ treatments, but by a smaller factor of between two and threefold in comparison with the control hairy roots. 6,6′-Dimethoxygossypol levels were statistically greater with MeJ exposure in some experiments, but not statistically greater in other experiments, and the net increase in concentration over the control cultures was much lower than for the other gossypol compounds. Biochemically, methylation occurs by the transfer of methyl groups from *S*-adenosyl-L-methanonine to hemi-desoxygossypol (Liu et al. 1999). After conversion to hemi-gossypol, the compound is dimerized to form gossypol (Mao et al. 2006), and the presence of methyl-hemi-gossypol within the hemi-gossypol pool results in the formation of the mono- and di-methylated gossypol forms. The shift in the distribution of methylated gossypol forms in the elicited cultures indicates that the pool of hemi-gossypol is less proportionally methylated, suggesting that MeJ treatment does not augment the methylation reaction to the same degree as the other reactions of the synthesis pathway.

A number of questions remain to be answered about the control of the enantiomeric composition of gossypol in cotton plants that can vary markedly among different species, varieties and tissues. Gossypol enantiomer ratios have been reported for field grown root and seed tissue of the cotton variety used to initiate these cultures (Dowd and Pelitire 2008). In the hairy roots, the relative amounts of the enantiomers differed not only between gossypol, 6-methoxygossypol and 6,6′-dimethoxygossypol but also between elicited and control hairy roots (Table 2). This later observation suggests that MeJ elicitation may influence the mechanisms that control this ratio. From protein fractions from *G. hirsutum* var. *marie-galante* flowers that contain a large enantiomeric excess of the (+)-gossypol form, Liu et al. (2008) reported that the distribution of gossypol enantiomers appeared to be controlled by at least one and maybe more dirigent proteins. Furthermore, Zhu et al. (2007) obtained two upregulated dirigent-like cDNA clones from *G. barbadense* tissues challenged with a fungal agent. The observed differences in enantiomer ratios suggest that these dirigent proteins or their interaction with enzymes in the gossypol biosynthetic pathway are also affected by MeJ treatment.

This work has shown that MeJ is a useful elicitor of gossypol production in cotton hairy roots. Research-grade gossypol is typically isolated from seed, roots, or by-products of the oil extraction process, where starting concentrations are typically between 1 and 4%. Because much higher concentrations of gossypol-related compounds can be induced in hairy roots, these cultures appear to be a good source for future preparation of these materials. Additional advantages of this route of preparation include straightforward isolation of the terpene aldehydes due to the lack of fatty materials in the roots as compared with seed or oil refining-derived soapstock. Also, the ability to develop a steady supply of cultured root-like material eliminates the need to rely on field-produced plants or by-products derived from cottonseed processing. The presence of the methylated gossypol forms may be advantageous if these derivatives are sought; however, their presence may also interfere with the recovery of a "pure" gossypol product. This issue is being addressed by hairy root transformation of cultivars of *G. arboreum*, an old-world cotton species that is reported to be void of the methylated gossypol forms (Stipanovic et al. 1975). Finally, introduction of appropriate precursors to the culture medium of cotton hairy roots allows the facile production of isotopically labeled products that would benefit current investigations on the mechanism of action of these compounds.

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References

Avancini G, Abreu IN, Saldaña MDA, Mohamed RS, Mazzafera P (2003) Induction of pilocarpine formation in jaborandi leaves by salicylic acid and methyljasmonate. Phytochemistry 63:171–175

Bulgakov VP, Tchernoded GK, Mischenko NP, Khodakovskaya MV, Glazunov VP, Radchenko SV, Zvereva EV, Fedoreyev SA, Zhuravlev YuN (2002) Effect of salicylic acid, methyl jasmonate, ethephon and cantharidin on anthraquinone production by *Rubia cordifolia* callus cultures transformed with the *rolB* and *rolC* genes. J Biotechnol 97:213–221

Conceição LFR, Ferreres F, Tavares RM, Dias ACP (2006) Induction of phenolic compounds in *Hypericum perforatum* L. cells by Colletotrichum gloeosporioides elicitation. Phytochemistry 67: 149–155

Dodou K (2005) Investigations on gossypol: past and present developments. Expert Opin Investig Drugs 14:1419–1434

Dowd MK, Pelitire SM (2006) Isolation of 6-methoxy gossypol and 6,6′-dimethoxy gossypol from *Gossypium barbadense* Sea Island cotton. J Agric Food Chem 54:3265–3270

Dowd MK, Pelitire SM (2008) HPLC preparation of the chiral forms of 6-methoxy gossypol and 6,6′-dimethoxy gossypol. J Chromatogr B 867:69–77

Durrant WE, Dong X (2004) Systemic acquired resistance. Annu Rev Phytopathol 42:185–209

Guillon S, Trémouillaux-Guiller J, Pati PK, Rideau M, Gantet P (2006) Hairy root research: recent scenario and exciting prospects. Curr Opin Plant Biol 9:341–346

Hron RJ Sr, Kuk MS, Abraham G (1990) Determination of free and total gossypol by high performance liquid chromatography. J Am Oil Chem Soc 67:182–187

Hron RJ Sr, Kim HL, Calhoun MC, Fisher GS (1999) Determination of (+)-, (−)-, and total gossypol in cottonseed by high-performance liquid chromatography. J Am Oil Chem Soc 76:1351–1355

Hunter RE, Halloin JM, Veech JA, Carter WW (1978) Exudation of terpenoids by cotton roots. Plant Soil 50:237–240

Kang S-M, Jung H-Y, Kang Y-M, Yun D-J, Bahk J-D, Yang J-K, Choi M-S (2004) Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. Plant Sci 166:745–751

Kim HL, Calhoun MC, Stipanovic RD (1996) Accumulation of gossypol enantiomers in ovine tissues. Comp Biochem Physiol 113B:417–420

Lee K-T, Hirano H, Yamakawa T, Kodama T, Igarashi Y, Shimomura K (2001) Responses of transformed root culture of *Atropa belladonna* to salicylic acid stress. J Biosci Bioeng 91:586–589

Li Y, Kiddle G, Bennett R, Doughty K, Wallsgrove R (1999) Variation in the glucosinolate content of vegetative tissues of Chinese lines of *Brassica napus* L. Ann Appl Biol 134:131–136

Liu J, Benedict CR, Stipanovic RD, Bell AA (1999) Purification and characterization of *S*-adenosyl-L-methionine: Desoxyhemigossypol-6-*O*-methyltransferase from cotton plants. An enzyme capable of methylating the defense terpenoids of cotton. Plant Physiol 121:1017–1024

Liu J, Stipanovic RD, Bell AA, Puckhaber LS, Magill CW (2008) Stereoselective coupling of hemigossypol to form (+)-gossypol in moco cotton is mediated by a dirigent protein. Phytochemistry 69:3038–3042

Mace ME, Bell AA, Stipanovic RD (1974) Histochemistry and isolation of gossypol and related terpenoids in roots of cotton seedlings. Phytopathology 64:1297–1302

Mao Y-B, Lu S, Wang L-J Chen X-Y (2006) Biosynthesis of gossypol in cotton. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 1:12

Mikkelsen MD, Petersen BL, Glawischnig E, Jensen AB, Andreasson E, Halkier BA (2003) Modulation of CYP79 genes and glucosinolate profiles in *Arabidopsis* by defense signaling pathways. Plant Physiol 131:298–308

Peñuelas J, Llusià J, Filella I (2007) Methyl salicylate fumigation increases monoterpene emission rates. Biol Plant 51:372–376

Pieterse CMJ, van Loon LC (1999) Salicylic acid-independent plant defense pathways. Trends Plant Sci 4:52–58

Royce HD, Harrison JR, Hahn ER (1941) Cotton-root bark as a source of gossypol. Oil Soap 18:27–29

SAS Institute Inc (1999) StatView reference, 3rd edn. SAS Institute Inc, Cary

Shanks JV, Morgan J (1999) Plant 'hairy root' culture. Curr Opin Plant Biol 10:151–155

Sirvent T, Gibson D (2002) Induction of hypericins and hyperforin in *Hypericum perforatum* L. in response to biotic and chemical elicitors. Physiol Mol Plant Pathol 60:311–320

Smith FH (1961) Biosynthesis of gossypol by excised cotton roots. Nature 192:888–889

Stipanovic RD, Bell AA, Mace ME, Howell CR (1975) Antimicrobial terpenoids of gossypium: 6-Methoxygossypol and 6,6′-dimethoxygossypol. Phytochemistry 14:1077–1081

Stipanovic RD, Puckhaber LS, Bell AA, Percival AE, Jacobs J (2005) Occurrence of (+)- and (−)-gossypol in wild species of cotton and in *Gossypium hirsutum* var. *marie-galante* (Watt). J Agric Food Chem 53:6266–6271

Suresh B, Thimmaraju R, Bhagyalakshmi N, Ravishankar GA (2004) Polyamine and methyl jasmonate-influenced enhancement of betalaine production in hairy root cultures of *Beta vulgaris* grown in a bubble column reactor and studies on efflux of pigments. Process Biochem 39:2091–2096

Triplett BA, Moss SC, Bland JM, Dowd MK (2008) Induction of hairy root cultures from *Gossypium hirsutum* and *Gossypium barbadense* to produce gossypol and related compounds. In Vitro Cell Dev-Plant 44:508–517

Vander Jagt DL, Deck LM, Royer RE (2000) Gossypol: prototype of inhibitors targeted to dinucleotide folds. Curr Med Chem 7:479–498

Wang Y-D, Yuan Y-J, Wu J-C (2004) Induction studies of methyl jasmonate and salicylic acid on taxane production in suspension cultures of *Taxus chinensis* var. *mairei*. Biochem Eng J 19:259–265

Wang Y-D, Wu J-C, Yuan Y-J (2007) Salicylic acid-induced taxol production and isopentenyl pyrophosphate biosynthesis in suspension cultures of *Taxus chinensis* var. *mairei*. Cell Biol Int 31:1179–1183

Xu Y-H, Wang J-W, Wang S, Wang J-Y, Chen X-Y (2004) Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-δ-cadinene synthase-A. Plant Physiol 135:507–515

Xu L, Yang D, Wang S, Tang W, Liu M, Davis M, Chen J, Rae JM, Lawrence T, Lippman ME (2005) (−)-Gossypol enhances response to radiation therapy and results in tumor regression of human prostate cancer. Mol Cancer Ther 4:197–205

Yuan Y-J, Wei Z-J, Miao Z-Q, Wu J-C (2002) Acting paths of elicitors on Taxol biosynthesis pathway and their synergistic effects. Biochem Eng J 10:77–83

Zayed R, Wink M (2004) Induction of tropane alkaloid formation in transformed root cultures of *Brugmansia suaveolens* (Solanaceae). Z Naturforsch 59c:863–867

Zhu L, Zhang X, Tu L, Zeng F, Nie Y, Guo X (2007) Isolation and characterization of two novel dirigent-like genes highly induced in cotton (*Gossypium babadense* and *G. hirsutum*) after infection by *Vericillium dahliae*. J Plant Pathol 89:41–45